EMT, cancer stem cells and autophagy; The three main axes of metastasis

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ABSTRACT

Epithelial-mesenchymal transition (EMT) and Cancer stem-like cells (CSCs) are major factors contributing to the metastasis of cancer cells. Consequently, the signaling pathways involved in both processes are appropriate therapeutic targets in the treatment of metastasis. Autophagy is another process that has recently attracted the attention of many researchers; depending on the type of cancer and tissue and the stage of cancer, this process can play a dual role in the development of cancer cells. Studies on cancer cells have shown that different signaling pathways are involved in all three processes, namely, cancer stem cells, autophagy, and EMT. The purpose of this study was to investigate and elucidate the relationship between the effective signaling pathways in all three processes, which could play an effective role in determining appropriate therapeutic goals.

1. Introduction

It is important to understand the molecular mechanism of metastasis so as to select a suitable therapeutic target for cancer treatment. The term “metastasis”, as a hallmark of cancer, was used for the first time by Jean Claude in 1829 [1]. Metastasis is a Greek word meaning
“displacement”, with meta meaning “next” and stasis, “placement” [2]; this term refers to cells migration from the primary tumor to different parts of the body and the formation of new tumors, which is the main cause of cancer-related death [3]. One of the molecular mechanisms involved in cancer cell metastasis is the EMT process. Recent studies have reported that EMT can have a key role in cancer cell metastasis as well as drug resistance [4]. During EMT, cells lose cell-cell and cell-extracellular matrix adhesion, acquiring mesenchymal cell phenotypes. This allows the detachment of cells from the primary tumor and the separation of the surrounding tissues and distant organs. EMT process has a major part in embryonic morphogenesis, tissue fibrosis, wound healing, and cancer cells metastasis [5].

On the other hand, there is substantial evidence indicating that the metastasis of cancer cells begins from cells with the most characteristics of stem cells, called cancer stem cells (CSCs) [6]. CSCs are known as cells that show self-renewal ability and asymmetric division. These cells are also associated with cancer cell growths and metastasis and tumor recurrence following treatment [7]. Studies have recently shown that targeting CSCs can be an effective treatment strategy for cancer treatment [8].

Autophagy is a highly conserved catabolic process that plays a fundamental role in maintaining cellular homeostasis through the translocation of damaged organelles, pathogens, and aggregated proteins to lysosomes and their breakdown by lysosomes [9]. This process is significantly involved in the cellular response to stress conditions such as nutrient deprivation and other environmental stresses effective in cell survive [10,11]. Studies have recently reported that autophagy plays dual roles in tumor cells. In the primary stages of tumor progression, it acts as an inhibitor of tumorigenesis, but in the following stages, it is involved in the preservation, growth, and proliferation of tumor cells [10]. In tumor development stages, autophagy is able to keep tumor cells alive under environmental stresses, and it seemingly plays a fundamental role in tumor cell metastasis [11]. Despite the various studies that show the role of each of these processes in metastasis, there are few studies on the interaction of each of these processes with each other in promoting cancer cell metastasis. Therefore, the aim of this study was to investigate the interaction of these processes with each other in the induction of metastasis in cancer cells.

2. EMT and metastasis

Epithelial-to-mesenchymal transition (EMT) is a process during which epithelial cells acquire the phenotype of the mesenchymal stem cells. This process is involved in many fundamental processes, including
embryonic evolution, tissue formation, wound healing, and tissue fibrosis. Moreover, EMT can be effective in tumor cell growth, drug resistance, and proliferation of tumors. Importantly, a huge amount of literature has established that EMT has a part in the metastasis of tumor cells [12,13] (Fig. 1). Given its critical role in metastasis, EMT has attracted the attention of many researchers.

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Different signaling pathways can be involved in EMT: Transforming growth factor beta (TGF-β) signaling pathway, Bone morphogenetic protein (BMP) signaling pathway, Receptor tyrosine kinase (RTK) signaling pathway, Wnt/*β-catenin* signaling pathway, Notch signaling pathway, Hedgehog signaling pathway, Signal transducer and activator of transcription 3 (STAT3) signaling pathway, Extracellular matrix (ECM)-mediated signaling pathway, and Hypoxia signaling pathway (Fig. 2) [14–16]. These signaling pathways change the gene expression through modulating the main transcription factors such as Snail, Twist and ZEB. These pathways increase the expression of mesenchymal cell markers, reduce the epithelial cell markers, and finally change the phenotype of epithelial cells to mesenchymal stem cells [17].

Snails are a family of ZINC finger transcription factors involved in various molecular mechanisms. This family comprises three members, namely, Snail1 (Snail), Snail2 (Slug) and Snail3 (Smuc) [18,19]. One of the most important members of this family is Snail1 that has an important role in the induction of EMT in cancer cell migration and metastasis. In promoter Snail1, there exist different binding sites for several transcription factors such as Notch intracellular domain (NICD), Lysyl oxidase like 2 (LOXL2), Nuclear factor-κB (NF-κB), Hypoxia-inducible factor 1-alpha (HIF-1a), IκB kinase α (IKKα), SMAD, High mobility group AT-Hook 2 (HMGA2), Early growth response protein 1 (EGR-1), Poly [ADP-ribose] polymerase 1 (PARP-1), STAT3, Metastasis associated 1 family member 3(MTA3), and Glioma-associated oncogene homolog 1 (Gli1) which is able to directly bind to Snail promoter and increase the expression of Snail [20].

Recent studies have shown Snail1 to be involved in the EMT process through various pathways. This transcription factor has multiple binding sites on E-cadherin, a key gene implicated in EMT. The changes in the expression level of E-cadherin are closely associated with enhanced EMT and metastasis. Snail1 is also able to regulate the proteins involved in cell-cell interaction and cell structures such as Claudin (CLDN), Occludin (OCLN), Zona occludens 1 (ZO-1), Cytokeratin 18, and Mucin 1. Evidence further indicates that the expression of matrix metalloproteinases (MMP), especially MMP-2 and-9, is governed by Snail1. Importantly, studies have revealed that during EMT, Snail1 upregulates the expression of other transcription factors involved in EMT such as ZEB-1 and ZEB-2, thereby further inducing EMT [20,21]. Immunohistochemical studies on patients with prostate cancer show that Snail1 is highly expressed in the first steps of tumor formation [22].

Twist is the second effective transcription factor in EMT. This transcription factor belongs to the BASIC HELIX-LOOP-HELIX (BHLH) and plays a pivotal role in myriad physiological and pathological phenomena [23]. The Twist family consists of two members, including Twist-1 (Twist) and Twist-2 (Dermo-1) [24], having a key part in the development and formation of mesodermal layer. Mutation in this transcription factor in drosophila causes Twisted phenotype which is associated with a lack of internal organ. In humans, this mutation is associated with Saethre-Chotzen syndrome, where patients are faced with craniosgnostosis and mild limb anomalies [25]. Previous studies on Twist-1 reported that this protein has a critical role in the different steps of cancer progression such as metastasis, angiogenesis, and stemness. However, there is little information concerning the molecular basis of Twist-2 [24,25]. Studies on clinical samples show that high expression levels of Twist-1

are highly correlated with the invasion and migration of cancer cells. Moreover, in in-vitro studies, enhanced Twist-1 expression increases invasion and migration and anoikis resistance in cancer cells [26]. Therefore, based on evidence, increased expression of Twist-1 can facilitate the EMT process and lead to metastasis in cancer cells [27]. Zinc finger E-box-binding homeobox (ZEB) is known as the third major transcription factor that plays a fundamental role in the regulation of EMT. This family is comprised of two members, namely, ZEB1 and ZEB2 [28], which affect the expression of various genes involved in embryogenesis, differentiation, and also tumorigenesis and metastasis of cancer cells [29]. Studies have indicated that these two are key nuclear factors in the EMT process. ZEB1 and ZEB2 restrain the expression level of E-cadherin through binding to the E-BOX sequences of E-cadherin promoter, thereby playing a role in the induction of metastasis characteristics in cancer cells [28]. Furthermore, ZEB1 decreases epithelial cell properties and enhances metastasis via binding to the promoter of other effective target genes involved in cell-cell interaction, tight junctions (TJ), desmosomes, and cell polarity [29,30].

One of the main features of the EMT process in cancer cells is its anoikis resistance, which plays a key role in metastasis [31]. Anoikis is the programmed cell death by which epithelial cells become apoptotic by breaking from the extracellular matrix and their neighboring cells. This process acts as a barrier against metastasis. However, cancer cells resist anoikis due to molecular mechanisms during separation from the original sites and travelling in the circulatory and lymphatic system [32]. Numerous lines of evidence demonstrated that EMT-promoting proteins are one of the main inducers of anoikis resistance in human tumors. For example, decreasing E-cadherin expression and increasing N-cadherin act as an enhancer of anoikis resistance to invisibility and metastasis. Also, some studies show that EMT transcription factors such as Twist, Snail, Zeb1 have an effective role in anoikis resistance by acting on the expression E-cadherin and N-cadherin [33]. Induces

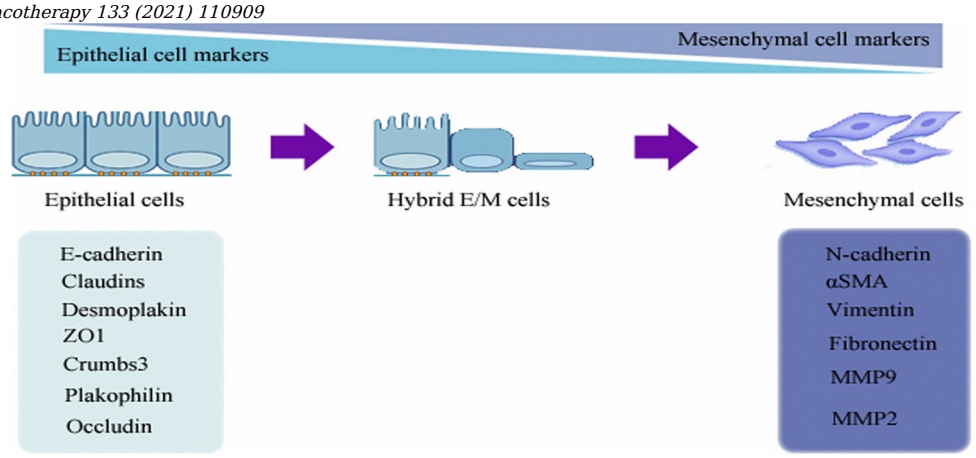

Fig. 1. An overview of the EMT process: in this process, the epithelial marker decreases, the mesenchymal marker increases, and the cells lose their polarity and are converted to the motile cells.

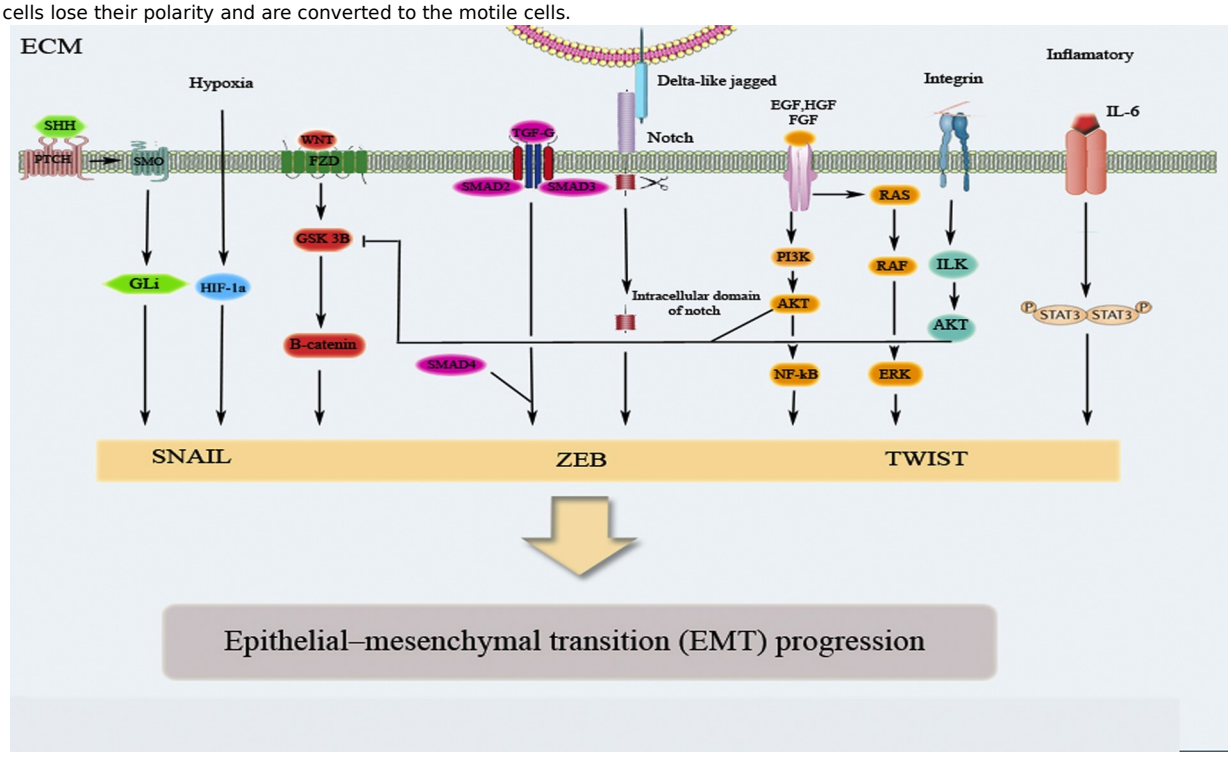

Fig. 2. An overview of signaling pathways effective in the EMT process.

metastasis and anoikis resistance [34]. Ankyrin-G protein is another link between E-cadherin which is resistant against anoikis. Ankyrin-G uses proteins such as E-cadherin to provide a bridge between the cytoskeleton and the cell membrane. This protein in plasma membranes separates a protein called NRAGE from translocation to the nucleus. During the EMT process, with the lack of E-cadmium and the absence of ankyrin-G, NRAGE is translocated to the nucleus and promotes anoikis resistance by reducing the tumor suppressor p14ARF which acts as an inducer of anoikis [31]. Also, some studies have provided evidence that the E-cadherin throughout the Hippo signaling pathway reinforces anoikis, the hemophilic binding of E-cadherin protein to two neighboring cells. In contrast, disruption of the interaction between E-cadherin and β-catenin with impaired YAP phosphorylation acts as a remarkable enhancer of anoikis resistance [31]. Studies also showed that N-cadherin through PI3K activated Akt and the PI3K / Akt signaling pathway, resulting in induced anoikis resistance in human tumors [34].

Due to the role of EMT in cancer cell metastasis, this process has attracted the attention of many researchers in the field of cancer treatment. For example, simvastatin is an FDA-approved drug which is used to treat hyperlipidemia. Evidence has shown that this compound acts as one of the inhibitors of EMT. Studies in cancers of the bladder, ovaries, and pancreas show that simvastatin inhibits metastasis and cell invasion by inhibiting the expression of factors involved in EMT, such as cadherin, vimentin, and β-catenin. It also acts as an EMT inhibitor in vivo. Various Phase II clinical trials use simvastatin as a drug for the treatment of advanced-stage carcinomas, and studies are ongoing [35]. Also, LY2157299 (galunisertib) is another EMT inhibitor that inhibits this process by

inhibiting TGF - β pathway, as one of the main inducers of EMT. This combination is in phase II clinical trials for the treatment of glioblastoma and hepatocellular carcinoma [36].

3. Autophagy and metastasis

Autophagy is regarded as self-degradation and conserved processes [37] that have pivotal parts in controlling the quality of cellular components and maintaining cellular homeostasis. This process is involved in turnover long-lived proteins, misfolded proteins, aggregated proteins, damaged organelles (peroxisomes, mitochondria, endoplasmic reticulum), and pathogens [37,38]. Under normal conditions, autophagy acts as a housekeeping process; however, under stress conditions such as nutrient deprivation, oxidative stress, hypoxia, and infection, autophagy provides the essential nutrients and adapted cells with stress conditions [38,39]. Recent studies have shown that autophagy is associated with the initiation and development of tumors [40] that Autophagy has also been reported to play a dual role in cancer cells. On a basic level, autophagy acts as a process that stabilizes the genome of cells and prevents the formation of tumor cells; when tumor cells are formed, however, autophagy plays an important role in the development and progression of cancers [38].

According to recent investigations, reduced expression of BECN1, a protein with a key role in phagophore formation and autophagy, is found to increase the growth and proliferation of many tumor cells, including the prostate, breast, and ovary. Therefore, based on the points mentioned above, it can be concluded that protein acts as a tumor suppressor. In contrast, other studies have reported that autophagy in advanced tumor stages entails the growth and proliferation of tumor cells. When tumor cells are faced with different stressing conditions, they activate autophagy so as to become compatible with those conditions. For instance, hypoxia in the cells of central tumors leads to the induction of autophagy in these cells. On the other hand, autophagy provides a high metabolic and energy requirement for tumor cells to grow and proliferate at advanced stages through autophagy induction and recycling of intracellular components [41]. In the metastasis of cancer cells, autophagy acts as a double-edged knife. In the first stages of metastasis, it can suppress metastasis. In the advanced stages, however, it contributes to metastasis through increasing the cell growth, proliferation, and cancer cell survival. In metastasis, in addition to overcoming environmental stresses such as hypoxia and nutrient deprivation, cells have to detach from the ECM and enter the bloodstream. When normal cells detach from ECM, they initiate a programmed cell death called anoikis, which inhibit metastasis. However, cancer cells are capable of escaping from anoikis when detached from the extracellular matrix [41,42]. Because of these biological effects, many scientists are looking to target this process to treat cancer. For example, Chloroquine and hydroxychloroquine FDA-approved drugs that act as inhibitors of autophagy in late-stage through disrupting the autophagosome-lysosome fusion stage [43]. Lys 05 is another derivative of chloroquine, which accumulates in lysosome and shows the strongest anti-autophagic and anti-cancer effects of HCQ. 3-Methyladenine (3-MA) is another inhibitor that exerts its effects in the early stages of autophagy. This compound inhibits autophagy by inhibiting class III phosphatidylinositol 3-kinase (PI3K) [43].

4. CSCs and metastasis

Stemness of cancer cells plays an important role in the growth, progression, and survival of tumor cells. This process primarily results in the self-renewal of cells; secondly, it allows the cells to generate differentiated cells. The interaction between stem cells and the environment is also effective in growth and proliferation. Stem cells utilize this property for tissue homeostasis while CSCs use this feature to maintain cell survival and malignancy [44].

Cancer cells are treated at an early stage whereas certain remaining tumor cells might be involved in tumor recurrence. These cells are called CSCs which are involved in treatment-resistant cancer cells and exhibit properties and functions similar to stem cells [45]. These cells allow for tumor growth in the early stages and play a pivotal role in the growth and development of new tumors [46]. CSCs are identifiable in the majority of frequent tumors such as liver, breast, prostate, head and neck, pancreas, leukemia, and melanoma. Various methods are employed to identify CSCs, often based on the identification of cell surface markers [47]. The most frequently studied CSC surface markers are CD44, CD24, CD29, CD90, CD133, epithelial-specific antigen (ESA), and aldehyde dehydrogenase 1 (ALDH1) which is used to identify and isolate these cells [48].

In various cancers, the self-renewal ability is not only stimulated but also constantly active. This characteristic of CSCs plays a critical role in the metastasis and invasion of tumor cells. CSCs are associated with the growth and proliferation of tumor cells via high cell turnover and generating precursor cells differentiated into various types of cells [44]. Metastasis of cancer cells is related to CSCs which migrate to distant organs and form new tumors. Studies have observed that in the early stages of metastasis, cancer cells have gene expression patterns similar to normal stem cells [49]. As noted above, inhibition of cancer stem cells can be an appropriate therapeutic strategy to inhibit metastasis. For example, Resveratrol is a polyphenolic compound that reduces the population of breast cancer stem cells in mice by inhibiting the Wnt / β-catenin signaling pathway [50]. It also reduces the expression of genes involved in stem cell and self-renewal capacity in human pancreatic cancer stem cells and KrasG12D mice [51]. Metformin is an FDA Approved drug used to treat type 2 diabetes, obesity, and polycystic ovarian syndrome. Evidence also shows that this compound selectively inhibits stem cells. Also, in the xenograft mouse model, metformin in combinatorial therapy with doxorubicin inhibits tumor mass and prevents relapses [52]. However, given the similarities

between cancer stem cells and normal human stem cells, there is a need for more extensive studies to identify specific targets and appropriate drugs to target cancer stem cells.

5. Crosslink between EMT and CSCs

The association between cancer stem cells and EMT has attracted the attention of many researchers because of the similar role that these two processes play in cancer cell metastasis. Evidence suggests that EMT, in addition to playing a pivotal role in the metastasis of human tumors, is closely related to the function of cancer stem cells. More recently, evidence has emerged that the cells acquired cancer stem-like properties under the influence of the EMT process, result in increased relapse, drug resistance, and metastasis in many human tumors [53] (Fig. 3). Growing evidence supports that there is an overlap between EMT stimuli and cancer stem cells. In addition, Inhibitor Agents whose primary purpose is to inhibit EMT, not only inhibit EMT and metastasis but also suppress the stem cell-like properties [53]. They have reported that EMT induction by different factors increases the expression level of genes involved in stemness and enhances the expression level of stem cell markers in immortalized epithelial cells, leading to metastasis in cancer cells [54]. When influenced by EMT, cancer cells acquire the same property of stem cells, implying the relationship between CSCs and EMT. In breast cancer, EMT induction increased the expression of stem cell gene profiles such as CD44 + / HIGH / CD24− / LOW [55].

The signaling pathways associated with CSCs and EMT have not been elucidated. However, accumulating evidence indicates that both processes are mediated by different signaling pathways, including *TGF*-β, *Wnt/β-catenin, Hedgehog, Notch,* and STAT3 [56,57]. Choi et al. reported that stem cell markers and EMT markers were closely correlated with regard to colorectal adenocarcinoma. They also showed that the expression level of EMT markers (E-cadherin, β-catenin, Snail, Vimentin) was correlated with CD133 expression level, cancer cell invasion, and metastasis [58]. Single et al. found that EMT induction by TGF-β / CsA in renal cell carcinoma cells (A498) decreased the expression of epithelial cell markers and increased the expression of mesenchymal markers. Their studies on stem cell markers and chemo-resistance showed that TGF-β / caspase enhanced the expression of markers effective in stem cell and drug resistance [59]. Moreover, Vijay et al. showed that in negative breast cancer triple cells, EMT increased the population of CSCs. They further reported that inhibition of GSK3B, as a regulator of the EMT process, decreased the expression level of mesenchymal cell markers and the CD44 $^{+}$ / CD24^{-} cell population [60]. In a study, Wang and colleagues reported that the Hedgehog signaling pathway induces EMT in pancreatic cancer stem cells. They observed that SMO knockdown caused the inhibition of EMT and suppressed the invasive ability of pancreatic cancer stem cells [61].

Literature survey about crosslinke between EMT and cancer stem cells revealed that transcription factors of EMT in cancer cells play a role in maintaining stem cells by inducing the expression of genes involving cancer stem cells. Vesna et al. Reported that over-expression of TWIST with Don regulation of CD24, induces cancer stem cell phenotype in breast cancer cells [62]. Lu et al. also reported that A549-Snail cells compared to A549-vector cells showed high-level expression of genes involved in stemness. On the other hand, the population of cancer cells with CD44high / CD24 low phenotype is high frequency in the A549-Snail cells. In addition, the expression level of CD 133 as a marker of cancer stem cells has increased [63]. Similar to this study, Zhou et al. reported that Snail is an effective factor in maintaining the stem cell-like phenotype in pancreatic cancer. Moreover, Snail silencing suppresses colony and spheroid formation, as well as reducing the expression of transcription factors and markers involved in CSCs [64]. Bhat-Nakshatri et al. reported that SLUG overexpression in breast cancer cells increased the frequency of the cell population with the CD44 + / CD24 + phenotype and also enhanced the ability of mammosphere forming breast cancer cells [65].

The relationship between EMT and stem cells is not a one-way street. Some studies report the influence of transcription factors involved in cancer stem cells in the regulation of EMT. For example, Wang and colleagues showed that Oct-4 and Nanog positively regulate the process of EMT and metastasis in breast cancer patients [66]. Also, R. Palla and colleagues demonstrated that Nanog by binding to the promoter of genes involved in EMT including Zeb1, Zeb2, Twist1, Prrx1 and miR-21, induces this process and metastasis in squamous cell carcinoma [67]. Gao et al. reported that SOX2 increased slug expression by activating the STAT3 / HIF-1α signaling pathway, thereby inducing the process of EMT and metastasis in esophageal squamous cell carcinoma (ESCC) [68].

Due to the close relationship between these two pathways in regulating each other and the role of these two pathways in cancer cell

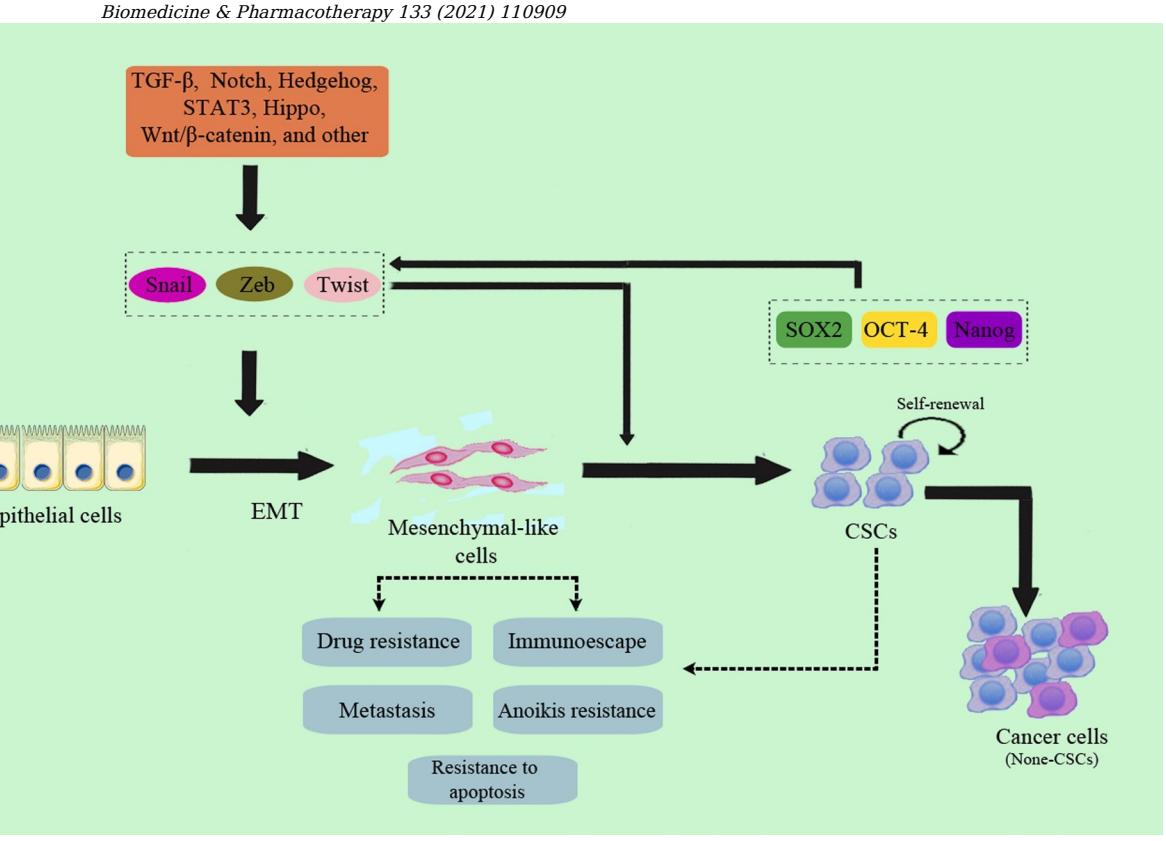

Fig. 3. An overview of the molecular mechanism of the relationship between autophagy, EMT, and CSCs.

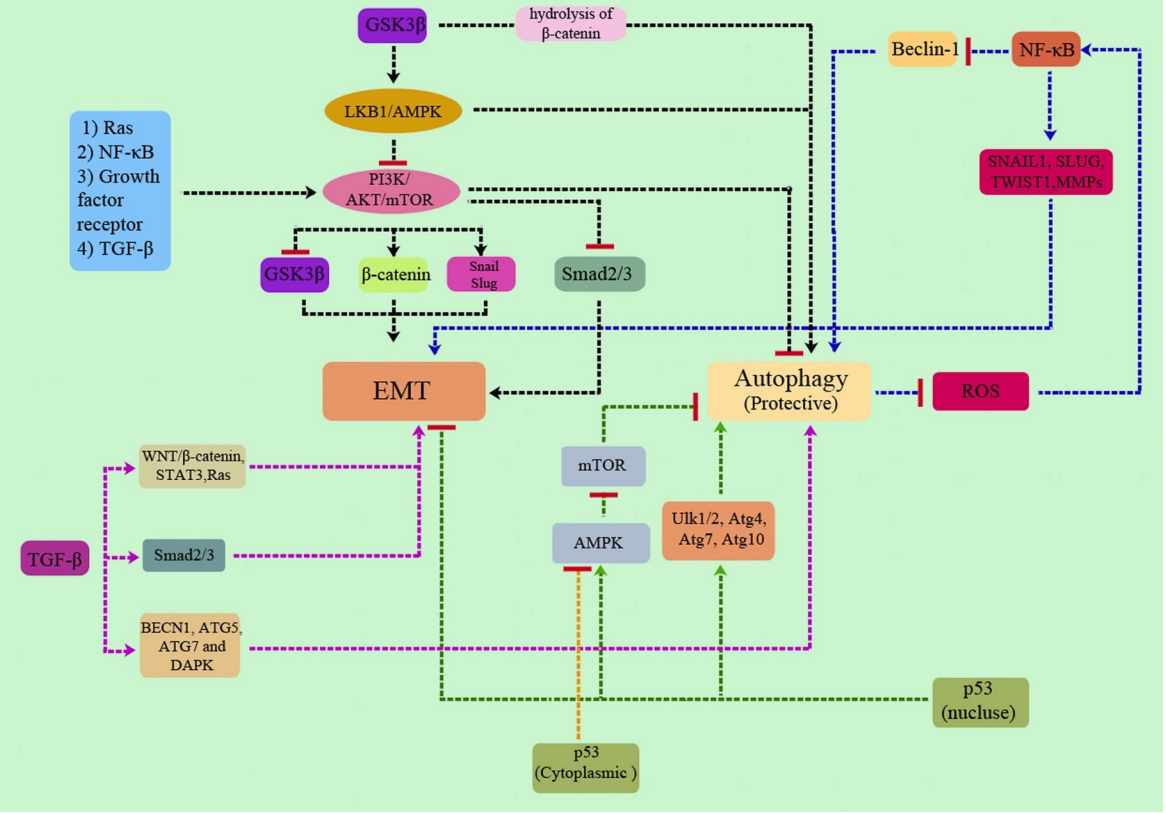

Fig. 4. An overview of the molecular mechanisms involved in crosslinks between EMT and Autophagy.

metastasis, common inhibitors of these two pathways can have a high therapeutic potential in inhibiting metastasis. In a study, Liang et al. reported that curcumin inhibited the EMT and CSCs properties in bladder cancer by inhibiting the Wnt / β-catenin signaling pathway [69]. Also, Wang et al. have shown that taxifolin (a flavonoid) inhibits EMT cell survival in class A549 and H1975 class through suppression stemness [70]. In another study, Cao et al. reported that Quercetin - 3 - methyl ether, by inhibiting the Notch1, PI3K / Akt, and EZH2 signaling pathways, simultaneously inhibited the EMT process and cancer stem cells, thereby inhibiting the invasion and metastasis of human breast cancer cells [71].

6. Crosslinks between EMT and autophagy

As stated above, the two processes of EMT and autophagy play a fundamental role in invasion and metastasis of cancer cells. According to the recent studies, there is a complex relationship between EMT-related signaling pathways and autophagy-related signaling pathways (Fig. 4).

One of the most important regulators of autophagy is mTOR. Under cellular stress condition, autophay is induced following the inhibition of mTOR; while under normal cellular condition (when nutrients are plentiful), mTOR inhibits ULK1 complex and autophagy by inhibiting ATG13 protein [72]. According to several pieces of evidence, different regulators such as AGC kinase family member Akt, serum/glucocorticoid regulated kinase (SGK), protein kinase C (PKC), and filamin A exert their effects by affecting mTOR. In this regard, PI3K/Akt/mTOR signaling pathway has attracted the attention of many researchers, and the upstream and downstream of this signaling pathway have been studied extensively. This signaling pathway is considerably regulated by LKB1/adenosine monophosphate–activated protein kinase (AMPK) and Ras in the upstream. Also, in the downstream, PI3K/Akt/mTOR signaling pathway affects p70 ribosomal S6 kinase (P70S6K) and eukaryotic initiation factor 4E (eIF4E) binding protein 1 (4EBP1), as the two major regulators of protein synthesis [73,74]. Recent studies have shown that in addition to induction of autophagy in cancer cells by inhibition of EMT, inhibition of mTOR can lead to inhibition of invasion and metastasis of cancer cells. For example, alteronol induces autophagy in cancer cells by inhibiting Akt/mTOR signaling pathway; on the other hand, it inhibits metastasis and invasion of cancer cells by inhibiting the process of EMT [75]. Three recent studies have reported that in pancreatic and ovarian cancer cells, Alisertib promotes autophagy by inhibiting phosphatidylinositol 3-kinase/Akt/mTOR signaling pathway, and on the other hand, it inhibits metastasis of cancer cells by inhibiting the process of EMT [76,77]. Recent studies suggest that this signaling pathway is hyperactivated in most cancer cells, and it has attracted the attention of many researchers [78]. In addition to inhibition of autophagy, activation of PI3K/Akt/mTOR signaling pathway leads to the induction of EMT in cancer cells. Several studies suggest that in the process of phosphorylation, Akt/mTOR signaling pathway induces EMT in cancer cells by activating the transcription factors involved in EMT. On the other hand, this signaling pathway decreases the expression of epithelial cell markers such as E-cadherin by inducing the expression of Twist and Snail and ZEB-1 [79]. Deng et al. reported that inhibition of PI3K/Akt/mTOR signaling pathway reverses the process of EMT and decreases the expression of mesenchymal cell markers [80]. Also, Chang et al. reported that in prostate cell lines that are resistant to radiation therapy, inhibition of PI3K/Akt/mTOR signaling pathway by dual PI3K/mTOR inhibitor (BEZ235) leads to inhibition of EMT and decreased expression of cancer stem cell markers [81]. However, studies suggest that EMT transcription factors can affect PI3K/Akt pathway. According to the recent studies, Twist can affect the Akt signaling pathway. This transcription factor enhances phosphorylation, invasion, and metastasis of cancer cells by increasing the expression of miR-10b. Also, the evidence suggests that by binding to E-box element in Akt2 promoter, Twist induces PI3K/Akt signaling pathway. Therefore, there is a positive feedback loop between Twist and Akt that leads to induction of the process of EMT and metastasis [82]. PI3K/Akt/mTOR signaling pathway is activated by different growth factors. For example, TGF-β is one of the most important EMT inducers and it is involved in induction of EMT by direct and indirect activation of PI3K/Akt/mTOR signaling [83,84]. This inductive effect can become reversed by the inhibitors of PI3K/Akt/mTOR signaling pathway, and it will lead to inhibition of metastasis [74].

Under energetic stress, AMPK-activated protein kinase (AMPK) plays the role of an energy sensor in cells, and it induces autophagy and energy homeostasis in cells by different mechanisms such as inhibition of mTOR. AMPK is activated by different molecular mechanisms, the most important of which is a tumor suppressor called Liver kinase B1 (LKB1), also known as serine/threonine kinase 11 (STK11). This protein exists in AMPK upstream, and Loss of LKB1 is involved in tumorigenesis and cancer progression in many cancers. In addition to indirect inhibition of autophagy by mTOR phosphorylation, AMPK directly inhibits autophagy by ULK1/Atg1 phosphorylation [85]. On the other hand, studies have revealed that in addition to inhibiting autophagy, LKB1/AMPK pathway negatively regulates cell invasion, too. According to studies, this signaling pathway is one of the inhibitors of the process of EMT. For example, activation of this signaling pathway by downregulation of the downstream factors of TGF-β signaling pathway such as MMPs and Snail leads to inhibition of cell migration [86]. Choudhury et al. reported that in prostate cancer cells, activators such as AICAR and A769662 inhibit cell migration by downregulating mTOR and P70S6Ki without affecting AKT [87]. The evidence suggests that LKB1/AMPK pathway is involved in inhibition of metastasis of cancer cells by affecting the other signaling pathways involved in EMT. Studies suggest that increased activity of AMPK is accompanied by decreased expression of glioma-

associated oncogene 1 (Gli1) [88]. By phosphorylation of serines 102 and 408 and threonine 1074 in Gli1, AMPK targets this protein to be decomposed by proteasomal and decreases its sustainability [89,90].

Over the past years, several studies have reported that NF-kB is one of the most important factors involved in invasion and metastasis of cancer cells. This transcription factor increases the expression of transcription factors involved in EMT such as SNAIL, SLUG, SIP1 and TWIST1; so, it induces EMT in cancer cells [91]. In addition to EMT, NF-kB is involved in stimulation and inhibition of autophagy by different mechanisms. According to the evidence, stimulation of NF-kB by TNF inhibits autophagy in most cancer cells; whereas, inhibition of NF-kB in these cells leads to reactivation of autophagy. It has been found that repression of autophagy in TNF-treated cells probably takes place by activation of authophagy inhibitor i.e. mTOR [74]. The inhibitory effect of NF-kB on autophagy has been proved in several studies. For instance, it has been found that inhibition of NF-kB in starved cells leads to induction of autophagy in these cells [92]. Also, the evidence suggests that NF-kB is also involved in activation of autophagy. According to the evidence, ROS is one of the factors involved in metastasis and invasion of cancer cells, and it exerts its effects by NF-κB–dependent autophagy. Studies performed in the recent years have revealed that inhibition of NF-κB–dependent autophagy makes the cancer cells sensitive to apoptosis [93,94]. On the other hand, induction of autophagy by inhibition of ROS–NF-κB signaling decreases the expression of MMP-2 and MMP-9, and it can be involved in inhibition of EMT [74,95].

Transforming growth factor-β (TGF-β) is another factor involved in the relationship between autophagy and EMT. This molecule is one of the potent inducers of the process of EMT that is highly expressed in most cancer cells [96]. According to studies, TGF-β signaling pathway can be involved in induction of EMT by both SMAD/non-SMAD pathways while directly affecting EMT-involved factors by induction of other signaling pathways such as NOTCH, WNT, INTEGRIN ، STAT3 [97]. In addition to induction of EMT, this molecule is also involved in induction of autophagy. Studies suggest that TGF-β induces autophagy by increasing the expression of autophagy-involved genes such as BECN1, ATG5, ATG7 and DAPK (death-associated protein kinase) [98]. Chao Zhang et al. reported that TGF-β2 induces autophagy in glioma cells by Smad and non-Smad pathways (JNK pathway). Also, the authors found that autophagy plays a fundamental role in induction of EMT by TGF-β. Induction of autophagy decreases the expression of EMT-related proteins and finally inhibits metastasis of cancer cells [99]. He et al. reported another pathway for induction of autophagy. They found that TGF-β plays a fundamental role in induction of autophagy and metastasis in prostate cancer cells by induction of transcription factor EB (TFEB) expression and activation of TGF-β-TFEB-RAB5A axis [100]. Hu et a.l reported that induction of autophagy by increased expression of TGF-β leads to induction of EMT in cancer cells. Mechanistically, induction of autophagy by decomposition of phosphodiesterase (PDE) 4A increases cAMP level in cells and activates PKA. The result will be activation of cAMP/PKA/CREB signaling pathway and increased expression of TGF-β1. Also, it was found that increased expression of TGF-β1 leads to induction of EMT and metastasis in cancer cells [101].

The other factor involved in the relationship between autophagy and EMT is P53. As a tumor suppressor, P53 plays a fundamental role in regulation of autophagy. In nucleus, P53 induces autophagy by increasing the expression of the genes involved in autophagy and inhibiting the PI3K/AKT/mTOR signaling pathway [74,102,103]. In contract, P53 has an opposite effect on cytoplasm and it inhibits autophagy. Under normal condition, P53 exists in cytoplasm and inhibits autophagy. However under cell stress, P53 is translocated from cytoplasm to nucleus and induces autophagy. According to studies, the inhibitory mechanism of cytoplasmic P53 is independent from its transcriptional function and is related to AMPK–mTOR signaling pathway. In cytoplasm, P53 inhibits the activity of AMP-dependent kinase, as one of the positive regulators of autophagy, activates mTOR, and inhibits autophagy [104]. In addition to autophagy, P53 is also involved in regulation of EMT. Recent studies have found that P53 inhibits EMT and metastasis by affecting the transcription factors involved in the process of EMT. Ren et al. reported that increased expression of WT-p53 by decreasing mesenchymal markers fibronectin, vimentin, N-cadherin, ZEB2 and increased expression of E-cadherin epithelial markers leads to inhibition of EMT and metastasis in prostate cancer cells [105]. According to the statements, it can be concluded that there is a complex relationship between autophagy and EMT in cancer cells. Therefore, inhibition of the process of EMT by controlling autophagy is a potential treatment strategy that should be studied further. Meanwhile, the relationship between these two processes should be extensively investigated in different cancers, so it will be possible to choose appropriate inhibitors or inducers of autophagy and provide the necessary conditions for inhibition of EMT and metastasis. In a study, Su et al. showed that autophagy and the EMT process promote cisplatin-resistance in nasopharyngeal carcinoma (NPC). Moreover, the Inhibition of autophagy by chloroquine (CQ) made the EMT process reversed [106]. Also, Jang et al. demonstrated that sinomenine hydrochloride (SH) reverses EMT and suppresses metastasis in human glioblastoma throught ER stress-induced autophagy [107].

7. Crosslinks between autophagy and CSCs in metastasis

A great deal of research has been documented that autophagy plays a key role in cancer cell metastasis. This process, with its protective role against oxidative stress, plays a central role in the survival of cancer stem cells. Evidence suggests that in cancer cells, autophagy acts as a double-edged sword. In addition, the details of the molecular mechanisms regulating protective or toxic (lethal)

autophagy are not clearly defined [108]. Accumulation of treatment-induced autophagosomes cannot determine toxic or protective autophagy function. In cancer biology, autophagy can be defined as "toxic" or "protective". Toxic autophagy, lethal (toxic) autophagy, or autophagic cell death (ACD) is defined as the type II programmed cell death, the induction of which can be an appropriate treatment approach to improve the treatment of apoptotic cancer cells. In contrast, the induction of protective autophagy leads to resistance against cell death-induced therapies. Inhibition of protective autophagy can be a treatment due to the sensitivity of tumor cells to activating apoptosis [109]. Autophagy (protective autophagy) is considered a major cause of survival and chemotherapy resistance in CSC (Fig. 5). It is also suggested that autophagy enables cancer cells to acquire CSC characteristics by inducing EMT and promoting the metastatic ability of human tumor cells. Thus, inhibition of autophagy is a promising way to overcome chemotherapy by resisting treatment strategies and thus destroying tumor cells by reducing tumor heterogeneity [110].

Over the past few years, numerous studies have investigated how protective autophagy is maintained by cancer stem cells. Recent studies have reported that autophagy is one of the key processes involved in maintaining stemness in both cancer and normal stem cells [111–113].

In recent years, researchers have attempted to clarify the molecular mechanisms of maintenance of cancer stem cells by the process of autophagy, and they have proposed various molecular mechanisms for maintenance of cancer stem cells by autophagy. For example, Paola Maycotte et al. reported that secretion of cytokines, especially IL-6, is one of the mechanisms by which autophagy maintains cancer stem cells. The authors have found that in autophagy-dependant cell line (MDA- MB-468), inhibition of autophagy by knockdown of ATG7 or BECN1 decreases the secretion of IL-6, cell survival, and mammosphere formation [114]. As a pleiotropic cytokine, high expression of IL-6 is observed in most cancers, and it is involved in proliferation, survival, invasiveness, metastasis, and maintenance of cancer stem cells by targeting and activating STAT3 signaling pathways [115]. In a study, Yoon et al. induced autophagy in Hela cells starved or treated with rapamycin to investigate the effect of autophagy on STAT3 signaling pathway. They found that induction of autophagy increases activation of STAT3, and inhibition of autophagy by 3-methyladenine inhibitingactivation of STAT3. Binding to IL-6 promoter, STAT3 increases its expression [116]. Therefore, it can be concluded that induction of autophagy increases the expression of IL-6 by activating the STAT3 signaling pathway, and it is involved in maintenance of cancer stem cells by increasing the activity of STAT3 signaling pathway.

Due to their high proliferation, tumors are always exposed to hypoxia which is accompanied by proliferation, metastasis, drug resistance, radiation therapy resistance, and recurrence of tumors. In this condition, expression of hypoxia-inducible factor 1α (HIF-1α) increases in cancer cells; this situation is involved in the cells' response to hypoxia by targeting special genes [117]. According to the recent studies, accumulation of cancer stem cells is usually found in hypoxic niches and hypoxia increases the self-renewal ability of these cells. Komal Qureshi-Baig et al. reported that hypoxia leads to phosphorylation of EZR at Thr567 by induction of autophagy and activation of PRKCA/PKCα; as a result, it increases the self-renewal capacity of cancer stem cells. They also reported that inhibition of autophagy by pharmacological methods or knockdown of the genes involved in autophagy inhibits the generation and progression of cancer cells by decreasing p-EZR [118].

A number of studies in recent years have suggested that autophagy is one of the responses of cancer cells to hypoxia, and it is induced by HIF- 1α. For example, Zhu et al. reported that induction of autophagy under hypoxia is effective in conversion of pancreatic cancer cells to stem-like cells by the aid of HIF-1α. Under hypoxia condition, compared to $CD133^-$ cells, density of $CD133^+$ cells increases as a marker of pancreatic cancer. This finding suggests that hypoxia probably induces the cancer stem cell properties in prostate cancer cells [119]. Zhu et al. found that pancreatic cells express a high metastasis capacity under hypoxia. In addition to expressing the stem cell properties, the migratory cells increase the expression of autophagy markers such as HIF-1α, LC3-II, and Beclin-1. On the other hand, inhibition of the expression of HIF-1α by siRNA inhibits the autophagy induced by hypoxia. Therefore, the results imply that hypoxia is probably involved in induction of autophagy and creation of stem cell properties by means of HIF-1α [120].

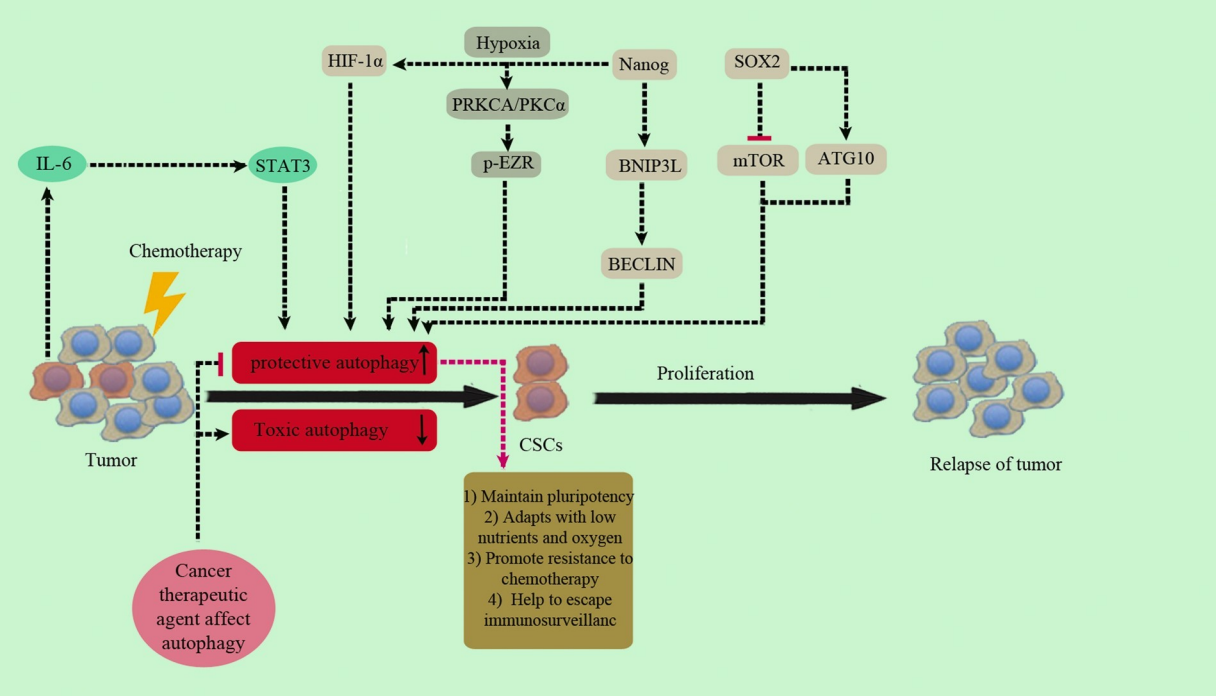

Fig. 5. An overview of the molecular mechanisms involved in crosslinks between autophagy and CSCs.

According to several studies, in addition to the role of autophagy in maintenance of cancer stem cells, some of the key transcription factors of cancer stem cells such as Nanog and Sox2 are involved in metastasis of cancer cells by induction of autophagy. For example, Hasmim et al. investigated the mechanism of the effect of Nanog on induction of autophagy under hypoxia; they found that Nanog directly binds to enhancer sequence in promoter of BNIP3L, as a protein releasing BECLIN from Bcl-2, increases its expression, and induces autophagy [121]. Shuo Wang et al. found that induced pluripotent stem cells (iPSCs) play a fundamental role in autophagy. Another study revealed that in these cells, Sox-2 binds to the promoter of mTOR, as an autophagy inhibitor, and decreases the expression of this protein; therefore, it induces autophagy in these cells [122]. As a similar finding, Cho et al. reported that in colon cancer cells, Sox2 induces autophagy. According to the evidence, Sox2 induces autophagy by binding to ATG10 promoter and affecting its expression [123]. In contrast, several studies have suggested the inhibitory effect of these factors on the process of autophagy. As an instance, Chou et al. studied A549 cells and found that Sox2 knockdown induces autophagy in these cells and causes accumulation of autophagic vacuoles in cytoplasm of these cells [124]. According to the points mentioned above, it can be concluded that autophagy is one of the factors maintaining cancer stem cells, and inhibition of autophagy can be a strategy for treatment of metastasis. Various studies show that the use of chemotherapy agents in combination with autophagy blockers can be used as a therapeutic strategy to increase the sensitivity of cancer stem cells. For instance, bevacizumab or temozolomide (TMZ) as an EGFR inhibitor combined with chloroquine as an end-stage inhibitor, increases cell sensitivity and reduces cell survival and

proliferation in glioblastoma cancer stem cells. Also, CQ and 5-fluorouracil in gastric cancer stem cells promote cell survivality [125]. In contrast, the induction of autophagy in cancer stem cells can also act as a treatment approach. Resveratrol reduces cancer stem cell markers in breast cancer stem cells by inducing autophagy. On the other hand, it inhibits cell survival and reduces tumor proliferation and volume in vivo [50]. Nevertheless, further studies should be done to recognize the molecular mechanisms related to the interaction of these two processes.

8. Conclusion

According to the results of this study, it can be concluded that EMT, CSCs, and the autophagy process are contributing factors in the metastasis of cancer cells. Accumulated evidence suggests that these three processes transform epithelial cells into motile cells with stem cell properties and resistance against chemotherapeutic agents. In this regard, evidence indicates that autophagy plays a role in regulating the EMT process and CSCs, essentially acting as a bridge which links the two processes. Given that, EMT, autophagy, and CSCs are the three main axes of metastasis. There are limited studies on the cross-linking molecular mechanisms of these three processes. Therefore, further studies on the overlapping molecular mechanisms of these three processes are necessary to identify a common therapeutic goal that simultaneously affects these three processes.

Declaration of Competing Interest

The authors reported no declarations of interest.

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